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Full Length Article

An Integrated Physical Map of the Cultivated Hot Chili Pepper, *Capsicum baccatum* var. Pendulum

Patricia M. Aguilera[1], Humberto J. Debat[2] and Mauro Grabiele[1*]
[1]*Instituto de Biología Subtropical, Universidad Nacional de Misiones (IBS-UNaM-CONICET), and Instituto de Biotecnología de Misiones, Universidad Nacional de Misiones (INBIOMIS-FCEQyN-UNaM), Posadas 3300, Misiones, Argentina*
[2]*Instituto de Patología Vegetal, Instituto Nacional de Tecnología Agropecuaria (IPAVE-CIAP-INTA), Córdoba X5020ICA, Argentina*
[*]For correspondance: maurograbiele@gmail.com; maurograbiele@conicet.gov.ar

Abstract

Capsicum baccatum var. Pendulum is the most frequently cultivated chili pepper in South America. This taxon is an outstanding source for breeding programs and genetic improvement in the genus, since its traits can be introgressed into *C. annuum* and related species to generate viable hybrids. In this study, the chromosome complement of *C. baccatum* var. Pendulum 2n = 24 was deeply characterized through a sequential combination of conventional and molecular cytogenetics approaches comprising Ag-NOR staining, heterochromatic fluorescence C-DAPI and CMA/DA/DAPI bandings and fluorescent *in situ* hybridization (FISH) of *Capsicum*-derived 5S and 18S-25S ribosomal genes and spacers probes. The attained markers were systematically integrated into a physical map which allows the identification of each individual chromosome. This is the first integrated physical map of *C. baccatum* var. Pendulum, which in conjunction with reported genetic linkage maps of *Capsicum*, would be useful for future high-density genetic mapping and whole-genome studies in *C. baccatum* and related taxa, as well as in breeding and genetic improvement programs in chili peppers. © 2017 Friends Science Publishers

Keywords: C-DAPI; CMA/DA/DAPI; 5S rDNA; 18S-25S rDNA; FISH; Heterochromatin

Introduction

Capsicum baccatum L. var. Pendulum is a hot chili pepper cultivated from the USA to Argentina and Chile, in addition to India (Moscone *et al.*, 2007). This domesticated form is the preferred pepper in Andean countries and is the most frequently grown *Capsicum* species in South America (Bosland and Votava, 2012). Its red or yellow fruits of particular hot taste are used for fresh consumption, in sauces and salads, as well as in processed spices or "Paprika". Often cultivated as ornamental plant, this species of high commercial value (Bosland, 1993) is also considered as a promising resource to the pharmaceutical industry (Zimmer *et al.*, 2012).

Besides, *C. baccatum* is an outstanding source for breeding programms for disease resistance in peppers. Several genotypes bearing tolerance/resistance have been reported in *C. baccatum*, *i.e.* to pepper fruit anthracnose (AVRDC, 1999; Park, 2005), to cucumber mosaic virus (Suzuki *et al.*, 2003) and particularly in var. Pendulum, to pepper yellow mosaic virus (Bento *et al.*, 2009). Since diseases can decrease mainly pepper yield and fruit quality, useful genes could be exchanged for improvement of these and other important agronomic traits. Moreover, those interesting features can be introgressed from *C. baccatum* into *C. annuum* L., which is the most commonly cultivated chili species worldwide, and related taxa from which fertile progenies are feasible to obtain (Pickersgill, 1991). Hence, the development of molecular markers and genetic maps are essential tools for pepper breeders in order to assist in the simultaneous selection of a particular array of genes/traits.

In this sense, in *C. baccatum* var. Pendulum a reference genetic map was recently developed (Moulin *et al.*, 2015). This map was based on 183 molecular markers, including SSR, ISSR and RAPD markers, mapped onto 16 linkage groups (12 larger and six smaller). Sixty one small-effect QTL, associated with 11 selected quantitative interesting agronomic traits, were detected in nine of these linkage groups (Moulin *et al.*, 2015).

In contrast to that genetic map, and despite the several cytological analysis performed in distinct accessions of *C. baccatum* var. Pendulum (Moscone *et al.*, 2007; Scaldaferro *et al.*, 2016), an integrated physical map of constitutive heterochromatic regions (Het), ribosomal loci (rDNA) and nucleolar organizer regions (NORs) in a single accession of this variety is unavailable, that could help to correlate chromosomes with genetic linkage maps and the future whole-genome sequence of the species.

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The main goal of this work was to obtain the first integrated physical map of *C. baccatum* var. Pendulum, through a combination of cytological approaches, allowing the identification of each chromosome of this variety. The integrated physical map presented here, in conjuction with the already existing genetic linkage map, would be useful for future high-density genetic mapping and whole-genome studies in *C. baccatum* and related taxa as well as in breeding and genetic improvement programs.

Materials and Methods

Plant Material

Fruits of *C. baccatum* L. variety Pendulum (Willd.) Eshbaugh, were acquired at a marketplace in the locality of Salta, Department Capital, Province of Salta, Argentina. Plants from germinated seeds cultivated at the campus of the Universidad Nacional de Misiones, in the locality of Posadas, Argentina, were subjected to taxonomical identification according to characters considered by Eshbaugh (1970).

Chromosome Preparations

Mitotic pretreatment and fixation of root tips and chromosome preparations for subsequent Ag-NOR staining, heterochromatic fluorescent bandings and FISH followed to Moscone *et al.* (1996a).

Ag-NOR Staining

Ag-NOR staining to reveal the active NORs in metaphase chromosomes was carried out as described by Stack *et al.* (1991) with few modifications.

Heterochromatic Bandings

Fluorescent chromosome banding to reveal the type, amount, size and distribution of Het was performed according to the triple staining technique (CDD) of Schweizer and Ambros (1994), using the fluorochromes chromomycin A3 (CMA), distamycin A (DA) and 4-6-diamidino-2-phenylindole (DAPI). CMA is specific to GC-rich Het while DAPI is specific to AT-rich Het. Additionaly, C-DAPI banding to reveal total Het was carried out following C-banding protocol of Schwarzacher *et al.* (1980) with the modification introduced by Lambrou and Ehrendorfer (2000) that includes a final staining with DAPI instead of Giemsa. The symbols "+" or "–" are used here to designate increased or decreased fluorescence, respectively.

Fluorescence *in situ* Hybridization

To analyze the intimate structure of the 18S-25S (45S) rDNA loci, different rDNA probes for FISH were developed (Grabiele, 2010) and assayed. Altogether, their originality reside in that they derive from chili peppers and cover the entire length of the rDNA unit in *Capsicum* (7.8 Kbp; Grabiele *et al.*, 2012), including genes, internal transcribed spacers (ITS) and the intergenic spacer (IGS). The 25S and 18S probes comprise the entire rDNA gene sequences (3.2 and 1.8 Kbp, respectively), while 5.8S/ITS probe (0.7 Kbp) involves the 5.8S gene and its flanking ITS_1 and ITS_2. The IGS4 probe (0.5 Kbp) consists of structural regions (SR) III-VI of the rDNA B-type IGS (2 Kbp) of *Capsicum* and includes the putative transcription initiation site motive (TIS) and diverse postulated regulatory elements widely conserved in Solanaceae. In addition, the IGS3 probe (1.8 Kbp) is a non-functional IGS variant from *Capsicum* complementary to IGS4, that lacks those regions and displays a longer SRII repetitive block. A complete description and discussion of *Capsicum* IGS regulatory regions are depicted in Grabiele *et al.* (2012). Nucleotide sequence data and supplementary information are available at the NCBI under the accession numbers FJ460246, FJ460247, JF766708, JF766709, JF766710 and JF766711. Furthermore, the production of the 5S rDNA *Capsicum*-derived probe (0.3 Kbp) for FISH is detailed in Aguilera *et al.* (2016). FISH experiments were undertaken according to Moscone *et al.* (1996b). The distinct rDNA probes were labeled by nick translation with digoxigenin-11-dUTP or biotin-11-dUTP following the manufacturer instructions (Enzo, USA). Slides preparations were subjected to RNAse and Proteinase K pretreatments, followed by steps of denaturalization, probe hybridization, blocking, probe detection by means of antibodies linked to fluorochromes [anti-digoxigenin to fluorescein (FITC) and anti-biotin to rhodamine (TRITC)] (Dako, USA), washing and DAPI staining for contrast. The appearance of DAPI enhanced regions subsequent to FISH procedure and DAPI counterstaining (FISH DAPI+ bands), that in fact mimic the C-banding pattern, is described in Moscone *et al.* (1999).

Fluorescence Microscopy and Image Acquisition

Chromosomes were viewed and photographed with a Leica DMLB fluorescence microscope (DMLB, Leica Microsystems, Wetzlar, Germany) equipped with a computer-assisted digital camera system. Images were captured in black and white using appropriate filters for FITC, TRITC and DAPI excitation, respectively. Digital images were combined in Photoshop CS6 (Adobe, USA) for final processing.

Karyotype Analysis

At least ten metaphase plates from three individuals were considered in the chromosome measurements. The centromeric index (CI) was used to classify the chromosomes according to Levan *et al.* (1964) in metacentric (m) and submetacentric (sm). Karyotype symmetry was calculated according to indexes A_1 and A_2 (Romero Zarco, 1986), r>2 and R (Stebbins, 1971), AI

(Paszko, 2006) and also CI. Different lengths of the same arm (and band/locus, where applicable) from homologous chromosomes were combined to mean values and then represented in the haploid complement of the idiogram. Intercalary markers were mapped using the index di = d x 100/a (d, distance of band center from the centromere; a, length of the corresponding chromosome arm) according to Greilhuber and Speta (1976).

Results

The accession of *C. baccatum* var. Pendulum analyzed here displays 2n = 24 median size chromosomes with a mean length of 6.19±0.67 µm and 74.25±5.50 µm per haploid genome. The karyotype, 22 m + 2 sm, is decidedly unimodal (A_2 = 0.10; R = 1.53) and symmetrical (A_1 = 0.17; r>2 = 0.08; CI = 44.99) and belongs to the category 2A of Stebbins and AI = 1.26 of Paszko. Pairs nos. 1, 3, 10 and 12 exhibit a secondary constriction associated to a terminal macrosatellite in their short arms and carry the active-NORs (Fig. 1A).

CMA/DA/DAPI fluorescent banding revealed a total lack of $DAPI^+$ regions (Fig. 1B) and confirmed 32 terminal CMA^+ sites (Fig. 1C). C-DAPI fluorescent banding unveiled several $C\text{-}DAPI^+$ bands, located at the whole centromere complement, intercalarly at the large arm of sm pair no. 12, and at each chromosome end, 32 of which co-localize with the CMA^+ regions (Fig. 1D).

The overall 18S-25S rDNA probes demonstrated comparable metaphase FISH patterns and revealed up to 32 terminal signals that include those expected at the active-NOR pairs nos. 1, 3, 10 and 12 (Fig. 1E, F, H, I; Fig. 2D, E, F). The 18S-25S rDNA appear as condensed blocks at interphase nuclei, mostly arranged at the periphery of the nucleus, though a small number of them locate within the nucleolus or at the nucleolar periphery, probably corresponding to the terminal macrosatellite of active-NOR pairs (Fig. 1E, F). At prometaphase, 18S-25S rDNA sites also arrange peripherically and adjacent to each other (Fig. 2 A). Co-localization of the entire 18S-25S rDNA loci with related $C\text{-}DAPI^+$ bands (Fig. 1D, G) and $FISH\text{-}DAPI^+$ bands which emulate the $C\text{-}DAPI^+$ banding pattern (Fig. 2B, C) is also evident. Double FISH of 18S-25S and 5S rDNA probes revealed an intercalar 5S locus at the short arm of pair no. 5, syntenic with a distal inactive-NOR rDNA site (Fig. 1H; Fig. 2D).

Obtained molecular cytogenetics markers for the accession of *C. baccatum* var. Pendulum analyzed here are summarized in Table 1. Integrated karyotype features and morphometric parameters of chromosomes are shown in Fig. 2G.

Discussion

Fish analysis with the distinct 18S-25S rDNA probes demonstrated comparable patterns considering the number and position of loci, which denoted that each locus is composed by the entire ribosomal unit. Remarkably, the 18S-25S rDNA signals far exceed in number to the active-NOR pairs. In this sense, extra NOR-inactive 18S-25S rDNA loci appear as non-functional. Co-localization of the whole 18S-25S rDNA loci with related CMA^+, $C\text{-}DAPI^+$ and $FISH\text{-}DAPI^+$ bands confirmed the heterochromatinized state of all the GC-rich rDNA sites, which explain both, the apparent inactivity of those extra rDNA loci and the expected inactivity of the NOR-associated Het portion of pairs nos. 1, 3, 10 and 12. The heterochromatic state of the entire 18S-25S rDNA loci is also evident at interphase nuclei, appearing as condensed blocks. According to the hypothesis of Kovarik *et al.* (2008), the distinct NOR behavior among 18S-25S rDNA loci in *C. baccatum* var. Pendulum-actives or inactives- may be related to structural and/or epigenetic divergence in those loci.

Two major structurally different Het fractions are recognized in variety Pendulum: one of them, represented by the terminal GC-rich blocks related to active-NOR- or inactive-NOR 18S-25S rDNA; the other, the blocks at centromeres, terminal regions and intercalarly at q12,

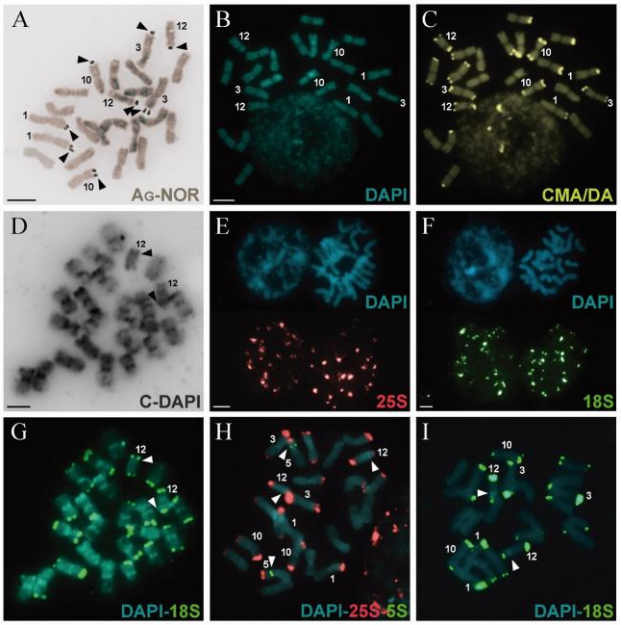

Fig. 1: Conventional and molecular cytogenetics characterization of *C. baccatum* var. Pendulum. A: Ag-NOR staining. B, C: DAPI and CMA/DA fluorescence bandings, respectively. D, G: C-DAPI fluorescence banding (colour-inverted) and subsequent FISH with 18S rDNA probe, respectively. E, F: FISH of 25S and 18S rDNA probes, respectively. H: Double FISH of 25S and 5S rDNA probes. I: FISH of 18S rDNA probe. Numbers and arrowheads point out the satellited active-NOR pairs nos. 1, 3, 10 and 12, the 5S ribosomal pair no. 5 and the intercalar band at q12. Scale bars = 5 µm; A, H and I share the scale

Table 1: Summary of conventional and molecular cytogenetics markers in *C. baccatum* var. Pendulum

Markers	Number and position of loci*
CMA/DA$^+$	26 subtelomeric regular loci (p1, 3, 5, 8, 10, 12; q3, 4, 6, 8, 9, 10, 12); 6 subtelomeric irregular minor loci (p2, 7; q5)
DAPI$^+$	-
C-DAPI$^+$	48 subtelomeric; 24 centromeric; 2 interstitial (q12), di=77.71
FISH-DAPI$^+$	48 subtelomeric; 24 centromeric; 2 interstitial (q12), di=77.71
5S rDNA	2 intersitial (p5), di=70.03
45S rDNA/active-NOR	8 subtelomeric (p1, 3, 10, 12)
45S rDNA/inactive-NOR	18 subtelomeric regular loci (p5, 8; q3, 4, 6, 8, 9, 10, 12); 6 subtelomeric irregular minor loci (p2, 7; q5)

*According to diploid genome. Numbers between brackets refer to chromosome pairs. p = short chromosome arm; q = large chromosome arm; 45S rDNA category includes different assayed probes that cover the entire ribosomal unit

which do not co-localize with 18S-25S rDNA sequences and thus possess a dissimilar origin. Unlike the CMA$^+$, C-DAPI$^+$ and FISH-DAPI$^+$ loci which are ribosomal in nature, the molecular foundation of those C-DAPI$^+$ and FISH-DAPI$^+$ heterochromatic sites distributed terminally, at centromeres and intercalarly at q12 remains unknown. In this sense, equivalent distal and pericentromeric heterochromatin, centromeres and interstitial knobs of tomato are composed by particular satellite DNAs and retroelements (Chang *et al.*, 2008), and in *C. annuum* and related taxa, the *Del* subgroup of *Gypsy* retroelements associates to heterochromatic regions (Qin *et al.*, 2014). In conjunction, the equilocal distribution at metaphase, the particular arrangement at prometaphase and interphase, and the condensed state throughout the cell cycle, illustrate the mode of dispersion of the distinct heterochromatic fractions in variety Pendulum in the terms defined by Schweizer and Loidl (1987).

The number and the interstitial position of the 5S locus are conserved throughout chili peppers (Aguilera *et al.*, 2016). The 5S rDNA locus in our plant material in fact co-localizes with an intercalar constitutive heterochromatic CMA$^+$ band at p5 of distinct accesions of *C. baccatum* var. Pendulum (Moscone *et al.*, 1996a); given the repetitive nature of the former, both markers converge at the same region.

Considering the genetic linkage maps on the *C. annuum* complex taxa of Wu *et al.* (2009) and Wu and Tanksley (2010), additional considerations can be made on *C. baccatum* var. Pendulum. In the context of the integrated physical map presented here, the chromosome pair no. 5 that carries the 5S rDNA actually correspond to the linkage group P1, and the 18S-25S rDNA active-NOR chromosome pairs nos. 10 and 12 associate to the linkage groups P2 and P8, respectively. Our findings are significant since the rDNA loci are not fully sequenced, assembled and anchored to related pseudomolecules in the projects embracing the *C. annuum* complex taxa yet (Kim *et al.*, 2014; Qin *et al.*, 2014) owing to the complexity in the analysis of repetitive DNAs.

Conclusion

The cytological approach used here allowed to obtain the first integrated physical map of *C. baccatum* var. Pendulum.

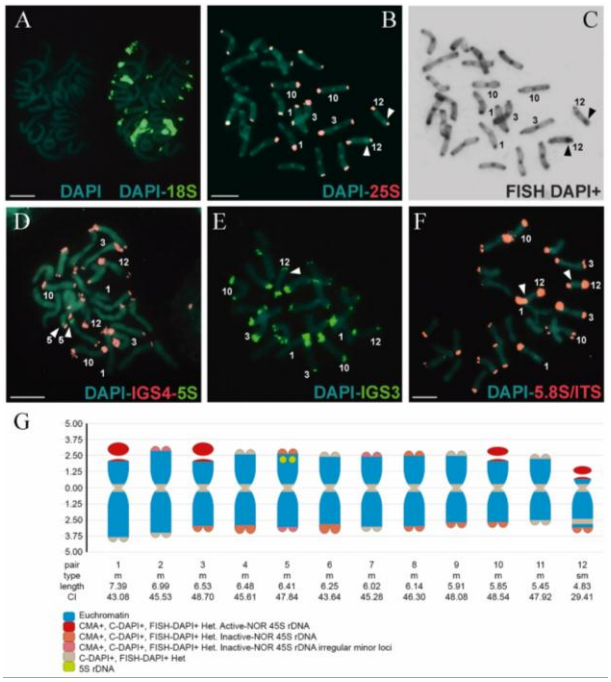

Fig. 2: Conventional and molecular cytogenetics characterization of *C. baccatum* var. Pendulum. A: prometaphase, FISH of 18S rDNA probe. B, C: FISH of 25S rDNA probe and identical spread deprived of rDNA signals and colour-inverted, respectively. D: Double FISH of IGS4 and 5S rDNA probes. E: FISH of IGS3 rDNA probe F: FISH of 5.8S/ITS rDNA probe. G: Integrated karyotype features and morphometric parameters of chromosomes. Numbers and arrowheads point out the satellited active-NOR pairs nos. 1, 3, 10 and 12, the 5S ribosomal pair no. 5 and the intercalar band at q12. Scale bars = 5 µm in A-F and 10 µm in G; B and E share the scale

This map is an arrangement of chromosomal and repetitive markers in which each metaphase chromosome can be accurately identified. This is a major goal ever since in species lacking whole-genome sequences and high-density genetic maps, such as *C. baccatum* var. Pendulum, basic physical maps are instrumental to integrate the incipient available information, as the reference genetic linkage map already existing for this taxon. The first integrated physical

map of *C. baccatum* var. Pendulum is now available as a reference karyotype for this important cultivated taxon as well as in breeding and genetic improvement programs in chili peppers.

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